Microtubule-dependent Control of Cell Shape and Pseudopodial Activity Is Inhibited by the Antibody to Kinesin Motor Domain

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Abstract. One of the major functions of cytoplasmic microtubules is their involvement in maintenance of asymmetric cell shape. Microtubules were considered to perform this function working as rigid structural elements. At the same time, microtubules play a critical role in intracellular organelle transport, and this fact raises the possibility that the involvement of microtubules in maintenance of cell shape may be mediated by directed transport of certain cellular components to a limited area of the cell surface (e.g., to the leading edge) rather than by their functioning as a mechanical support. To test this hypothesis we microinjected cultured human fibroblasts with the antibody (called HD antibody) raised against kinesin motor domain highly conserved among the different members of kinesin superfamily. As was shown before, this antibody inhibits kinesin-dependent microtubule gliding in vitro and interferes with a number of microtubule-dependent transport processes in living cells. Preimmune IgG fraction was used for control experiments. Injections of fibroblasts with HD antibody but not with preimmune IgG significantly reduced their asymmetry, resulting in loss of long processes and elongated cell shape. In addition, antibody injection suppressed pseudopodial activity at the leading edge of fibroblasts moving into an experimentally made wound. Analysis of membrane organelle distribution showed that kinesin antibody induced clustering of mitochondria in perinuclear region and their withdrawal from peripheral parts of the cytoplasm. HD antibody does not affect either density or distribution of cytoplasmic microtubules. The results of our experiments show that many changes of phenotype induced in cells by microtubule-depolymerizing agents can be mimicked by the inhibition of motor proteins, and therefore microtubule functions in maintaining of the cell shape and polarity are mediated by motor proteins rather than by being provided by rigidity of tubulin polymer itself.

MAINTENANCE of elongated shape and polarized pseudopodial activity of many cultured cell types, including fibroblasts and neurons, depends on the presence of an intact microtubule system. This dependence has been demonstrated in experiments with microtubule-depolymerizing drugs such as colchicine, colcemid, or nocodazole; fibroblasts treated with these drugs lose their elongated shape and become unable to move directionally (Vasiliev et al., 1970; Gail and Boone, 1971; Goldman, 1971). Distribution of pseudopodial activities at the edges of drug-treated fibroblasts is also significantly changed: stable zones of the edge disappear and the whole cell perimeter becomes active. However, the pseudopodial activity per unit length of active edge in the drug-treated fibroblasts is decreased significantly as compared to that of the control cells (Bershadsky et al., 1991). These results suggest that one of the functions of the microtubule system is to enhance or localize pseudopodial activity at one particular part of the cell, the leading edge, resulting in the acquisition of the extended cell shape and directed cell migration. This enhancement may be mediated by transport toward the cell edges to the plus ends of microtubules of certain cellular components required for the leading edge activity.

Alternatively, microtubules running parallel to stable cell edges may act as a rigid structural element which supports cell asymmetry by directly counteracting compression mediated by the actin cytoskeleton as proposed by Dennerll et al.

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(1988), or by restricting formation of membrane protrusions at the edges parallel to microtubules.

In this paper we present evidence that microtubule-dependent control of cell polarity in fibroblasts is in fact mediated, at least partially, by motor proteins, and therefore microtubules actively induce the activity of the leading edge rather than restrict it in the stable zones.

Involvement of microtubules in intracellular transport of organelles has been a subject of recent extensive studies (for review see Schroer and Sheetz, 1991) and microtubule-dependent motor proteins are thought to be responsible for organelle transport along microtubules. One of these proteins, kinesin, appears to be the most abundant translocator responsible for microtubule-dependent transport toward the cell periphery. The kinesin molecule is composed of two 60–80-kD light chains and two 110–130-kD heavy chains (Bloom et al., 1988; Kuznetsov et al., 1988). Kinesin heavy chain consists of a tail domain, a head or motor domain containing ATPase, and microtubule-binding sites (Hirokawa et al., 1989; Kuznetsov et al., 1989; Scholey et al., 1989; Yang et al., 1989). Kinesin is a member of a large superfamily of proteins that share closely related motor domains but are highly divergent outside this region (for reviews see Endow, 1991; Goldstein, 1991).

Recently, we raised a polyclonal antibody against the motor domain of *Drosophila melanogaster* kinesin heavy chain. This antibody, termed HD, recognizes kinesin polypeptides in cell and tissue extracts from various vertebrates (Rodionov et al., 1991; Gyoeva and Gelfand, 1991) and inhibits kinesin-driven gliding of microtubules in vitro (Rodionov et al., 1991). After injection into cells, antibody HD inhibits pigment granule dispersion in fish melanophores (Rodionov et al., 1991) and in coalignment of vimentin intermediate filaments with microtubules in fibroblasts (Gyoeva and Gelfand, 1991). In addition, injections of this antibody affect the formation of the mitotic spindle in cultured cells (Rodionov et al., 1993). These results have shown that we have obtained a powerful antibody probe suitable for investigating the function(s) of kinesin motors in a living cell. In the present work, we used microinjection of antibody HD to examine the role of motors in the control of shape and pseudopodial activity of normal human diploid fibroblasts and neurite formation in PC12 cells. Injection of HD antibody changed the shape and motility of fibroblasts in a way similar to the effect of microtubule-disrupting drugs. These results suggest that kinesin superfamily members are important mediators of microtubule-dependent control of cell shape and protrusive activity.

Materials and Methods

Materials

Unless otherwise indicated all chemicals were purchased from Sigma Immunochemicals (St. Louis, MO).

Cells

Normal human skin diploid fibroblasts, line 1036, were provided by Dr. V. Kuharenko (Institute of Medical Genetics, Russian Academy of Medical Sciences, Moscow). Cells between 15 and 25 passages were used in experiments described below. Fibroblasts were grown in Dulbecco's modified Eagle's medium (Flow Labs., Irvine, CA; ICN Biomedicals, Inc., Costa Mesa, CA) supplemented with 10% fetal calf serum and 5% postnatal human serum, penicillin (100 μg/ml), and streptomycin (100 μg/ml) at 37°C in 5% CO_2. For location of injected cells, coverslips with photo-etched locator grids (Bellco Biotechnology, Vineland, NJ) were used. For shape measurements, cells were plated at a low density (6×10^4 per cm^2) and incubated for 16–20 h before an experiment. Nocodazole was added to the tissue culture medium to a final concentration of 1 μg/ml and cells were incubated overnight.

Pseudopodial activity was measured at the leading edge of fibroblasts migrating into the wound. Cells were plated at a high density (about 5×10^5 per cm^2), grown for 2–3 d and the cultures were then wounded by removing part of the monolayer with a razor blade. Cells were then rinsed with growth medium carefully and incubated for additional 18–20 h before microinjection.

PC12 cells overexpressing gp140trk nerve growth factor receptor (Hempstead et al., 1992) were obtained from Dr. David Kaplan (Frederick Cancer Research Center, Frederick, MD) and maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 10% horse serum, penicillin (100 μg/ml), streptomycin (100 μg/ml), and 200 μg/ml of G418 antibiotic. Differentiation of these cells was induced by β subunit of nerve growth factor (10 ng/ml, Boehringer-Mannheim Corp., Indianapolis, IN) in serum-free medium for 12 h.

HD Antibody

Polyclonal affinity-purified antibody against the motor portion of *D. melanogaster* kinesin heavy chain (antibody HD) was produced in rabbit and purified as described previously (Rodionov et al., 1991). For control experiments, IgG were purified from the preimmune serum by chromatography on protein A–Superose column (Pharmacia Diagnostics Inc., Fairfield, NJ). Both control IgG and the HD antibody were dialyzed against a microinjection buffer containing 114 mM KCl, 20 mM NaCl, 3 mM $MgCl_2$, and 3 mM sodium phosphate buffer, pH 7.0, concentrated by ultrafiltration to 18–20 mg/ml, frozen in liquid nitrogen, and stored in aliquots at −70°C. Prior to an experiment, an aliquot of the antibody was thawed and centrifuged at 100,000 *g* for 30 min at 4°C to remove particulates.

Microinjection

Microinjection was performed as described previously (Rodionov et al., 1991) with micromanipulator (Carl Zeiss, Inc., Thornwood, NY) and 5242 Eppendorf microinjector using an ICM-405 inverted microscope (Carl Zeiss, Inc.) with 32× long working distance phase 1 lens. Cells were injected with glass micropipettes made from 1.2 mm OD capillaries containing internal filaments to aid loading (World Precision Instruments, Inc., Sarasota, FL). High concentration of antibody HD (see above) was used to perform gentle injections that did not exceed 5% of cell volume. Temperature was kept at 37°C during microinjection using a heating stage (Carl Zeiss, Inc.). Coverslips were then transferred into an atmosphere of 5% CO_2 and incubated for an additional 4 h before pseudopodial activity analysis or fixation for shape measurements.

Size and Shape Measurements

Size and shape of injected and noninjected fibroblasts were quantified as described by Dunn and Brown (1986) and Brown et al. (1989). Briefly, cells were washed with phosphate-buffered saline, fixed with formaldehyde (4%), and photographed using Photomicroscope III (Carl Zeiss, Inc.) equipped with the phase contrast optics. The outlines of cells were then drawn with the aid of photoenlarger and entered into a PC AT computer by tracing on a digitizing tablet (Summasketch II, Summagraphics, UK). Each outline consisted of approximately 100–200 coordinate pairs.

TRACER V1.0 software (Copyright Dr. Alastair Brown, King's College, London, UK) was used both for entering and storage of the outlines and for calculation of their shape characteristics; area, elongation, and dispersion. Elongation and dispersion were defined by Brown et al. (1989) as parameters that show how much the total mass of the shape extends away from its center of gravity, but elongation describes how much this extended mass can be reduced by compressing the shape to its center of gravity, while dispersion describes how much extended mass remains (see Dunn and Brown, 1986; Brown et al., 1989, for more details). Roughly, elongation can be considered as a measure of bipolarity, while dispersion a measure of multipolarity of cells. It was shown earlier that alterations of cell shape induced by microtubule-depolymerizing drugs can be adequately assessed by measurements of dispersion and elongation: both these measures are significantly decreased in the drug-treated fibroblasts as compared with control cells (Dunn and Brown, 1986; Middleton et al., 1989).

Pseudopodial Activity Measurements

Pseudopodial activity of fibroblasts migrating into an experimentally induced wound was measured as described in detail by Bershadsky et al. (1991). Coverslips were mounted in chambers filled with growth medium and analyzed at 37°C by video-enhanced DIC microscopy using Zeiss Axiophot microscope equipped with Hamamatsu C2400-01 Chalnicon videocamera (Hamamatsu Photonic Sys. Corp., Bridgewater, NJ). Images were processed with an Argus 100 Hamamatsu image analysis system and recorded with the Sony U-matic VO-7630 tape recorder (Sony, Montvale, NJ). Each cell was recorded for about 10 min. Cells were then fixed with formaldehyde (4%), permeabilized with 1% Triton X-100 in PBS, and immunostained with a fluorescein-conjugated goat anti-rabbit secondary antibody to reveal the injected antibody. Only cells proved to be injected by this method were further analyzed.

Four pairs of images separated by 20-s intervals were then selected from a 10-min record of each cell and the areas of protrusions and retractions were quantitated as described by Bershadsky et al. (1991). Briefly, the areas of protrusions and retractions per unit length of cell outline were calculated for each pair of images, the data for four pairs of images were averaged for each cell and the rates of protrusions and retractions were finally calculated as a change of average area per unit length of an outline per min.

Fluorescence Microscopy

For microtubule staining, cells were washed with PBS, permeabilized with 1% Triton X-100 in 50 mM imidazole-HCl buffer, pH 6.8, supplemented with 50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA, 1 mM β-mercaptoethanol, and 4% poly(ethylene)glycol 40,000 for 3 min, fixed with 1% glutaraldehyde, and after reduction with $NaBH_4$, stained with the monoclonal antibody DM1α against α-tubulin (Sigma Immunochemicals) and a fluorescein-conjugated goat anti-mouse antibody. For actin filament staining, cells were washed and extracted as described above, then fixed with 4% formaldehyde, and stained with rhodamine-phalloidin.

Injected cells were routinely identified after formaldehyde fixation by immunostaining for rabbit IgG with a rhodamine-conjugated goat anti-rabbit antibody.

For mitochondria staining, cells were incubated in a growth medium containing 10 μg/ml rhodamine-123 (Johnson et al., 1980), and then washed three times with a dye-free medium. Immunostained and rhodamine-123-stained cells were analyzed and photographed with a Photomicroscope III equipped with a condenser IIIRS.

Analytical Methods

For the preparation of cell extracts, fibroblasts were grown to confluency in a 100-mm Petri dish. Cells were washed three times with PBS containing the cocktail of protease inhibitors (1 mM PMSF, 10 μM leupeptin, 10 μM aprotinin, 10 μM pepstatin, and 10 μM soybean trypsin inhibitor), scraped off a dish and dissolved in 200 μl of an SDS sample buffer. SDS-gel electrophoresis was performed by the method of Laemmli (1970) in 4–12% gradient gels with acrylamide/methylenebisacrylamide ratio 30/0.8. Gels were stained with Coomassie blue R-250 or electroblotted to nitrocellulose (Towbin et al., 1979). Blots were successfully incubated with HD antibody (2 μg/ml) and affinity-purified goat anti-rabbit peroxidase-conjugated antibody (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD) diluted at 1:10,000. Peroxidase-labeled antibody was then detected by incubation of blots with the enhanced chemiluminescence (ECL)[1] reagents and exposure to the Hyperfilm ECL (Amersham Corporation). Partially purified HeLa kinesin (a gift of Dr. G. Hering) was used as a marker.

Results

Identification of Polypeptides, Recognized by HD Antibody in Human Fibroblasts

To determine whether microtubule-dependent control of shape and pseudopodial activity of fibroblasts is mediated by kinesin translocators in the present work we used antibody HD, raised against the *Drosophila* kinesin motor domain. It was important to check antibody specificity and to identify polypeptides that are recognized in human fibroblasts. We prepared cell extracts as described in Materials and Methods and used immunoblotting and sensitive ECL technique for the identification of immunoreactive polypeptides. Kinesin heavy chain partially purified from cultured HeLa cells was used as a marker. Fig. 1 shows the results of immunoblotting experiments. At a short exposure time of blots, kinesin heavy chain was the major polypeptide recognized by HD antibody in fibroblasts extract, though the antibody showed a weak reaction with additional lower molecular polypeptides. These polypeptides are likely to be the degradation products of kinesin heavy chain: the immunoreactive bands of the same electrophoretic mobility were found not only in cell extracts, but in the preparations of partially purified kinesin as well (Fig. 1, lane *c*). At longer exposure times additional polypeptides became visible and two of them had a relative molecular mass of 160 and 220 kD and thus could not be kinesin heavy chain degradation products (Fig. 1, lane *e*, *arrows*). These bands may correspond to kinesinlike proteins, though further work is needed for their identification as members of the kinesin family. The immunoblotting experiments thus showed high specificity of HD antibody used in our work: it bound in fibroblasts extracts with high affinity to the kinesin heavy chain and reacted weakly with additional bands that may correspond to the proteins of the kinesin family.

Antibody HD Interferes with Mitochondria Distribution in Human Fibroblasts

To determine whether kinesin motors are involved in maintenance of cell shape and directional pseudopodial activity of fibroblasts, we injected human diploid fibroblasts (line 1036) with antibody HD. This antibody has already shown to interfere with a number of microtubule motor functions, i.e., maintaining coalignment of intermediate vimentin filaments with microtubules in 1036 fibroblasts (Gyoeva and Gelfand, 1991) as well as centrifugal transport of pigment granules in fish melanophores (Rodionov et al., 1991). To check whether this antibody affected the intracellular transport of membranous organelles in fibroblasts as well, we analyzed the distribution of mitochondria in the cells in-

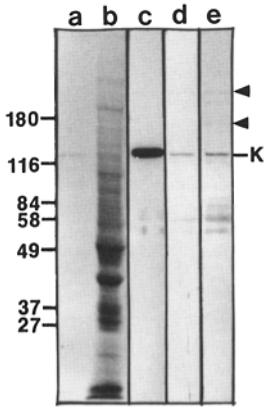

Figure 1. Polypeptides recognized by HD antibody in the extracts of human 1036 fibroblasts. Cell extracts and partially purified HeLa kinesin used as a standard were subjected to SDS-PAGE and gels were either stained with Coomassie blue, or electroblotted onto nitrocellulose membrane and stained with HD antibody and peroxidase-conjugated secondary antibody. Peroxidase labeled antibodies were detected by the ECL method. (*a* and *b*) Coomassie-stained gels; (*c–e*) fluorograms of the blot after exposure of the Hyperfilm ECL for 3 min (*d*) and 15 min (*c* and *e*). (*a* and *c*) HeLa kinesin; (*b*, *d*, and *e*) fibroblasts extracts. *K* indicates the position of kinesin heavy chain; arrows indicate the positions of minor high molecular weight bands recognized by HD antibody which can be seen after 15 min exposure of the Hyperfilm ECL (*e*).

1. *Abbreviation used in this paper*: ECL, enhanced chemiluminescence.

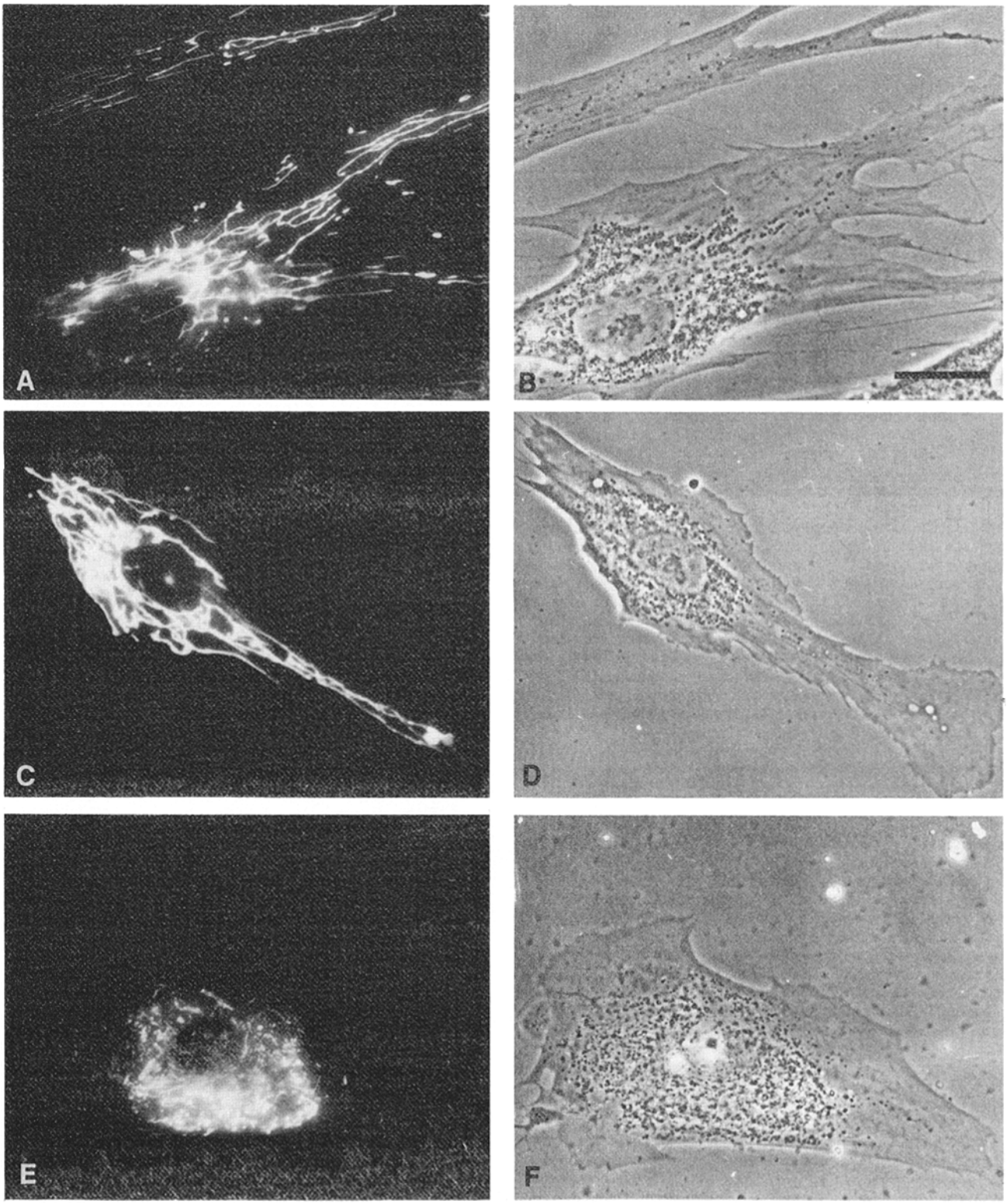

Figure 2. The effect of HD antibody injections on the distribution of mitochondria in 1036 human fibroblasts. Cells were injected with HD antibody or preimmune IgG at the concentration 20 mg/ml. After incubation for 4 h at 37°C, injected and noninjected cells, used as a control, were stained with rhodamine-123 to reveal mitochondria. *A* and *B*, non-injected cells; *C* and *D*, preimmune IgG–injected cells; *E* and *F*, HD-injected cells. *A*, *C*, and *E*, rhodamine-123 staining; *B*, *D*, and *F*, Phase contrast images. Individual mitochondria are not aligned in the plane of focus in *E* and therefore can not be fully displayed. Bar, 20 μm.

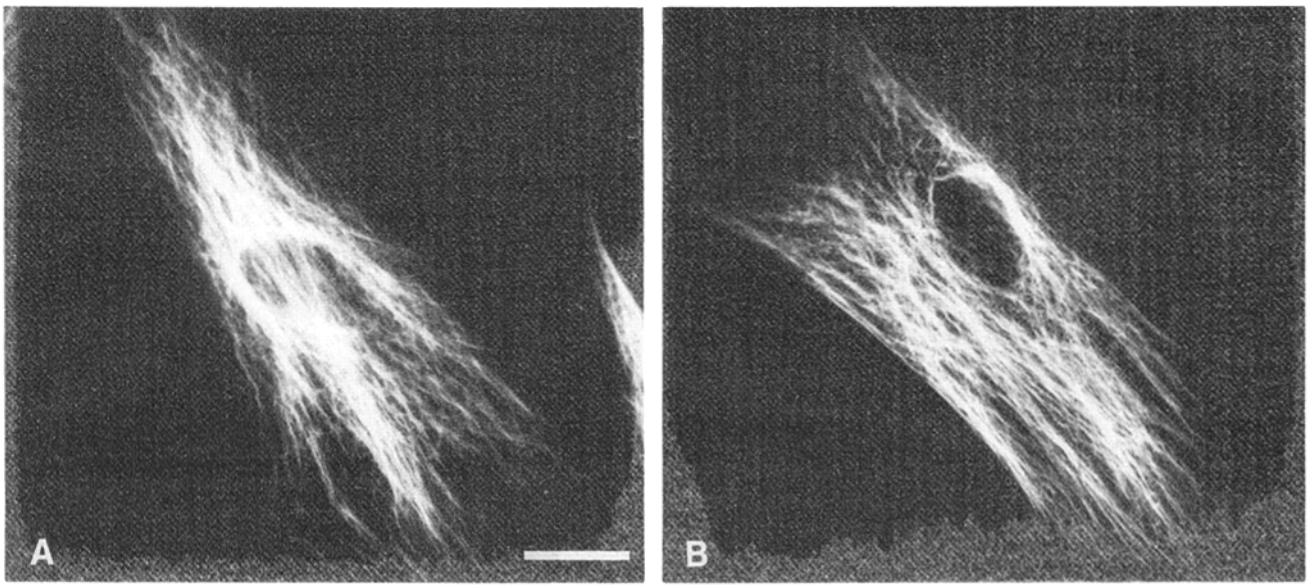

Figure 3. Microtubules in the fibroblasts injected with preimmune IgG and HD antibody. 1036 human fibroblasts were injected with either preimmune IgG (*A*) or HD antibody (*B*) at 20 mg/ml, incubated for 4 h at 37°C, fixed, and immunostained with the monoclonal antibody DM1α against α-tubulin and fluorescein-conjugated secondary antibody. Bar, 20 μm.

jected with the antibody. It is well known that mitochondria coalign with microtubules in fibroblasts and their distribution was found to depend on the integrity of the cytoplasmic microtubule network (Ball and Singer, 1982).

Fig. 2 shows the distribution of mitochondria revealed by rhodamine-123 staining in a noninjected cell, in a cell injected with preimmune IgG and in a HD-injected cell. In all control cells, long wormlike mitochondria extended from cell center and reached cell margins (Fig. 2 *A*). In contrast, in the cells injected with HD antibody, mitochondria formed bright fluorescent aggregates around the nucleus while the peripheral areas of cells were free from rhodamine-123 fluorescence (Fig. 2 *F*). Clustering in thick perinuclear parts of the injected cells made it difficult to visualize individual mitochondria in one optical section, but focusing through the cytoplasm showed that mitochondria are as long in the injected as in control cells, so no vesiculation of mitochondria was induced by HD antibody.

Immunofluorescence staining of these cells with anti-tubulin antibody showed that injection of HD did not change the distribution of microtubules (Fig. 3). Therefore, the effect of the injection on mitochondria distribution as well as any of the effects described below can not be explained by the destruction of microtubules.

Effects of HD Antibody on Fibroblast Shape

To investigate the effects of HD antibody on the shape of fibroblasts, cells grown in sparse cultures were injected with antibody HD or with preimmune IgG, fixed 4–5 h thereafter, and the two parameters, dispersions and elongations were measured.

Fig. 4 shows the typical outlines of cells obtained in these experiments. The injection of preimmune IgG did not influence the shape of the cells noticeably (compare Fig. 4, *A* and *B*). However, after injection of HD antibody, cells lost asymmetric morphology (Fig. 4 *C*) and their shape appeared to become similar to the shape of nocodazole-treated cells (Fig. 4 *D*).

Table I summarizes the results of quantitative analysis of the microinjection experiments. Though control injections slightly reduced the values of both dispersion and elongation, the HD antibody had a much more pronounced effect, inducing an almost 50% decrease in both parameters. The differences between HD antibody and preimmune IgG-injected cells in both dispersion and elongation were highly statistically significant, $P < 0.001$ in both cases according to Student's criterion. The effect of HD antibody on the fibroblast shape was very similar to the effect of nocodazole (Middleton et al., 1989; see also Table I, this paper). This effect could not be explained by nonspecific rounding of the cells, although microinjection per se sometimes, in fact, decreases cell-spreading area. It is important to note that preimmune IgG- and HD-injected cells did not differ significantly in the areas they occupy on the substrate. The mean area of noninjected cell measured in one of our experiments was 4,368 ±

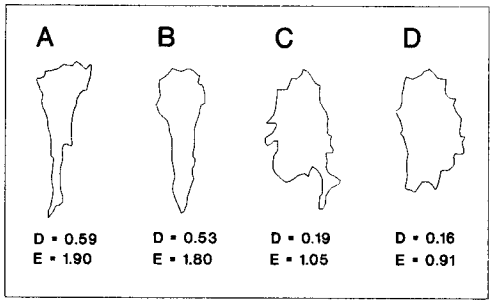

Figure 4. The typical outlines of noninjected (*A*), preimmune IgG-injected (*B*), HD-injected (*C*) and nocodazole-treated (*D*) fibroblasts. Cells with dispersion and elongation values close to the average values represented in Table I were selected and their outlines were drawn. Nocodazole treatment was for 12 h at 1 μg/ml.

Table I. Effect of Microinjections of Kinesin Antibody on Fibroblast Shape

	Control noninjected	Preimmune antibody	Kinesin antibody	Nocodazole treatment
Dispersion	0.54 ± 0.03	0.43 ± 0.03	0.27 ± 0.02	0.22 ± 0.02
Elongation	1.94 ± 0.06	1.67 ± 0.05	1.13 ± 0.04	0.90 ± 0.04
Number of cells	229	227	289	125

The cells were injected with either antibody HD or preimmune IgG, fixed 4-h later, and photographed using phase contrast microscopy. Nocodazole treatment was for 4 h at a concentration of 10 μM. Two parameters, elongation and dispersion (mean ± SEM) were determined for each cell as described in Materials and Methods.

338 (± SEM) μm^2, while the areas of preimmune IgG- and HD-injected cells were 3,965 ± 256 and 3,481 ± 208 μm^2, correspondingly; the difference between two last values is not statistically significant ($P > 0.1$) according to Student's t test. In addition, plotting of elongation and dispersion values versus surface area for preimmune IgG- and HD-injected cells revealed that the shape characteristics of cells with large area were also affected by HD antibody (not shown). So, HD antibody injections decreased both dispersion and elongation parameters of fibroblast cell shape; this effect could not be explained by nonspecific effects of injection itself.

Effects of HD Antibody on Leading Edge Activity and Actin Distribution in Migrating Fibroblasts

The experiments described above showed that antibody HD affected specifically cell shape and this effect was similar to the effect of microtubule-disrupting drugs. Another effect of microtubule depolymerization is the suppression of directional motility of fibroblasts and decreased pseudopodial activity at the leading edge of migrating cells (Bershadsky et al., 1991). To further compare the effects of HD antibody and microtubule disrupting agents, we tested whether the antibody had any effect on the pseudopodial activity at the leading edge of fibroblasts migrating into an experimentally induced wound.

Cells at the edge of the wound were injected with HD antibody or preimmune IgG and 4–5 h later analyzed with videoenhanced DIC microscopy and recorded on videotape. Noninjected fibroblasts were recorded as an additional control. Each cell was recorded for ~10 min and then pairs of successive images separated by 20-s intervals were selected randomly for further description and quantitation of pseudopodial activity as described in Materials and Methods.

Fig. 5 shows the leading edges of noninjected (*A* and *C*) and HD-injected (*B* and *D*) fibroblasts. Each pair of images was taken 20-s apart to illustrate protrusive activity of each cell type. Corresponding superimposed outlines of cell edges are shown on Fig. 5, *E* and *F*. Noninjected cells demonstrated wide lamella with rare microspikes. Ruffles were formed at the upper surface and moved centripetally. Mitochondria and membranous vesicles were usually abundant in the cytoplasm of the leading edge of intact fibroblasts. Preimmune IgG–injected cells looked very similar to intact cells (not shown). At the same time, HD-injected fibroblasts differed from preimmune IgG-injected and noninjected cells in both appearance of the leading edge and the level of pseudopodial activity. Microspikes were more numerous at the leading edge of HD-injected cells, than in the control cells (Fig. 5, *B* and *D*); their length and direction were constantly and rapidly changing. As was expected from the results of rhodamine-123 staining, mitochondria could not be seen at the leading edge. The overall pseudopodial activity at the leading edge of HD-injected fibroblasts was significantly reduced.

To quantitate the extent of suppression of pseudopodial activity in the HD-injected cells, the protrusion/retraction indices were measured for the leading edges of noninjected, preimmune IgG–injected, and HD-injected fibroblasts. The mean rates (μm/min) of protrusions and retractions at the leading edge of noninjected cells were 0.900 ± 0.112 and 0.766 ± 0.070 correspondingly (mean ± SEM). The values for preimmune antibody–injected cells were: 0.886 ± 0.078 and 0.670 ± 0.068. The same estimation for the cells injected by HD antibody revealed significant decrease of both mean protrusion (0.4180 ± 0.031) and retraction (0.415 ± 0.043) rates. Fig. 6 shows the histograms of protrusion/retraction indices obtained for 10 cells from each group. It is clear that preimmune IgG had only a minor if any effect on pseudopodial activity. At the same time, HD-injected cells displayed the suppression of motile activity at their leading edges; the indices of both protrusions and retractions were on average two times lower than in intact and preimmune IgG–injected fibroblasts. Thus, the HD antibody specifically inhibited protrusive activity at the leading edge of migrating fibroblasts. We think that inhibition of retraction observed in the injected cells is secondary to the inhibition of protrusion. In cultured cells most of protrusions of the pseudopodia are followed by their retraction and only the small fraction of protrusions lead to the attachment of pseudopodia to the substratum. Therefore, suppression of the protrusions should inhibit subsequent retractions, the type of response that, in fact, has been observed.

Since pseudopodial activity of a moving fibroblast is accompanied with the definite reorganization of the actin cytoskeleton at the leading edge (see Heath and Holifield, 1991, for a recent review), it was interesting to compare the actin distribution in antibody-injected and noninjected cells. Fig. 7 shows the results of rhodamine-phalloidin staining of the HD-injected (Fig. 7 *A*) and noninjected (Fig. 7 *B*) cells, migrating toward the wound. Both noninjected and preimmune IgG–injected (not shown) cells demonstrated typical distribution of actin polymer: distally to parallel stress fiber there was an actin-poor zone turning into the dense actin meshwork at the very margin of the leading edge (Fig. 7 *A*). In HD-injected cells, actin distribution was altered significantly: the actin-poor zone and distal meshwork were absent at the edge facing the wound, instead, abundant short bundles of actin filaments were located near the edge (Fig. 7 *B*, *arrows*). Similar changes were usually observed in nocodazole-treated cells (data not shown).

Effect of HD Antibody on Neurite Outgrowth

The results presented above show that the injection of the HD antibody into fibroblastic cells results in the changes of shape and motility that mimic those of the cells with microtubules destroyed by antimitotic drugs such as nocodazole or colcemid. Thus, although density or distribution of microtu-

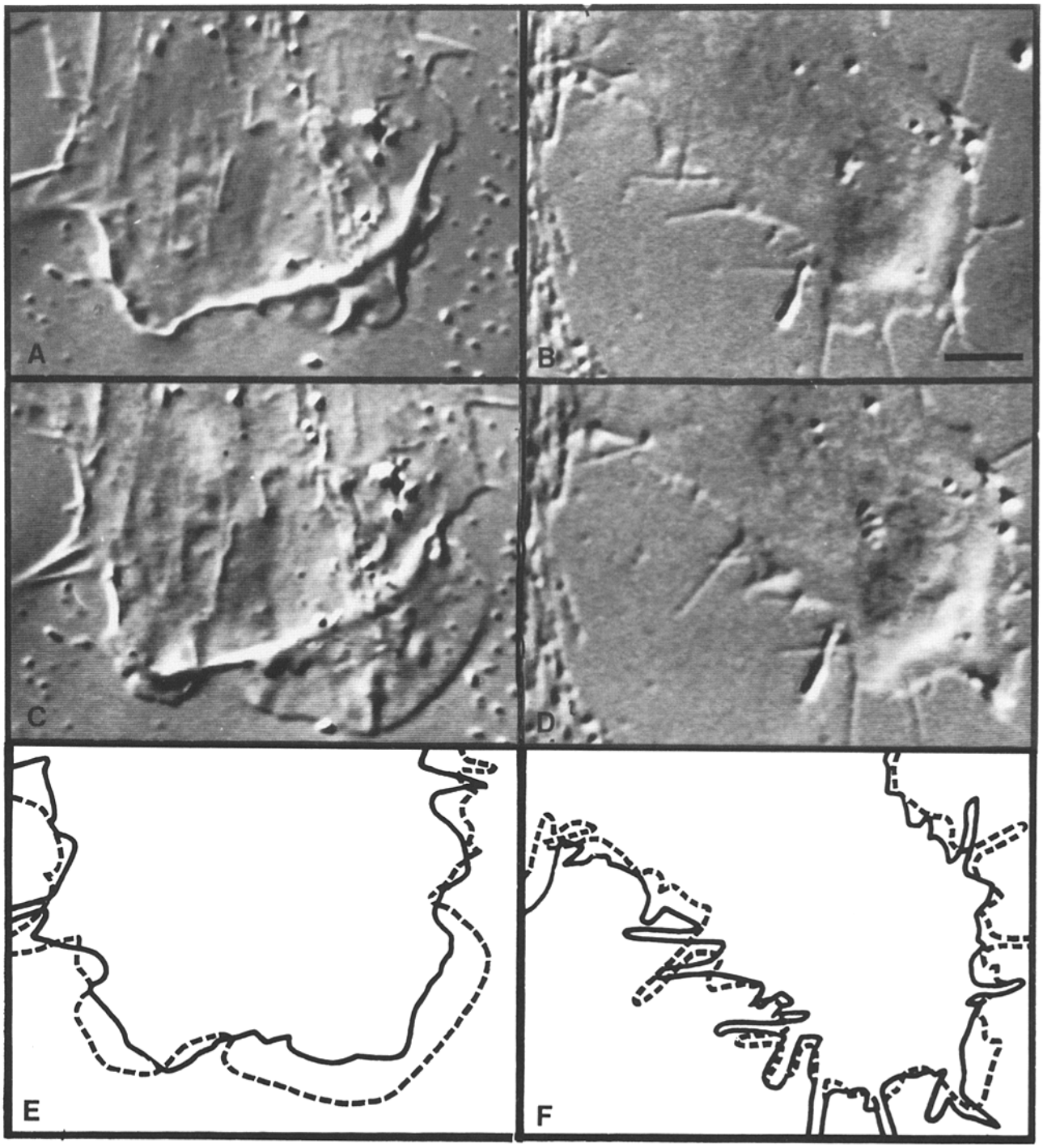

Figure 5. Effect of HD antibody injections on the pseudopodial activity at the leading edge of fibroblasts, moving into the experimentally induced wound. The fibroblast monolayer was scraped with a rubber policeman and 12–15 h later fibroblasts, actively moving into the wound, were injected with HD antibody (20 mg/ml). After an additional 4 h of incubation, the movements of the wound-facing edges of injected and control noninjected cells were observed with DIC optics and recorded on videotape. *A* and *C* and *B* and *D* are the pairs of images, separated by 20-s intervals. *E* and *F* show superimposed outlines of the leading edges shown correspondingly on *A* and *C* and *B* and *D*. *A*, *C*, and *E*, an intact cell; *B*, *D*, and *F*, a fibroblast, injected with the antibody. Bar, 5 μm.

bules in the injected cells was not changed as a result of the injection, inhibition of motor molecule(s) by the antibody prevents microtubule function in maintenance of fibroblast polarity. A more extreme case of microtubule-dependent polarization is provided by nerve cells, where the integrity of neurites depends on cytoplasmic microtubules (Joshi et al., 1985). To study the role of motors in maintenance of neurite growth and integrity, we injected the antibody into PC12 (Trk 6-24) pheochromocytoma cells (Hempstead et al., 1992) differentiated into neuronal morphology by the ad-

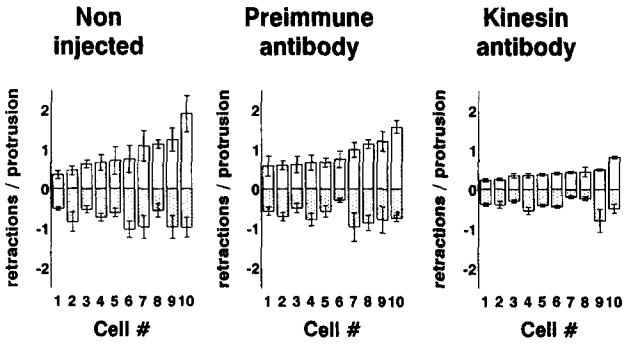

Figure 6. Histogram of protrusions and retractions of the leading lamella in control, preimmune IgG-injected and kinesin antibody-injected fibroblasts. Each bar represents an individual protrusion or retraction index.

dition of the nerve growth factor. We found that injection of the antibody into differentiated PC12 cells did not affect either neurite length or overall morphology of the cells even after prolonged (up to 6 h) observation. Moreover, the average rate of neurite outgrowth was not significantly changed as a result of the injection (9.7 ± 8.1 μm/h for injected and 10.3 ± 8.6 μm/h for noninjected cells). At the same time, immunofluorescent staining of the injected cells with a fluorescein-conjugated antibody against rabbit IgG shows that even after a 1-h incubation HD-antibody penetrated into all the neurites of the injected cells and spread all over the length up to the growth cones (Fig. 8).

We tried also to inject the antibody into undifferentiated PC12 cells, differentiate them for 8–12 h with NGF, and measure the number of cells that developed neurites with a length of more than three cell diameters. The results of the measurement show that about 75% of both control and injected cells

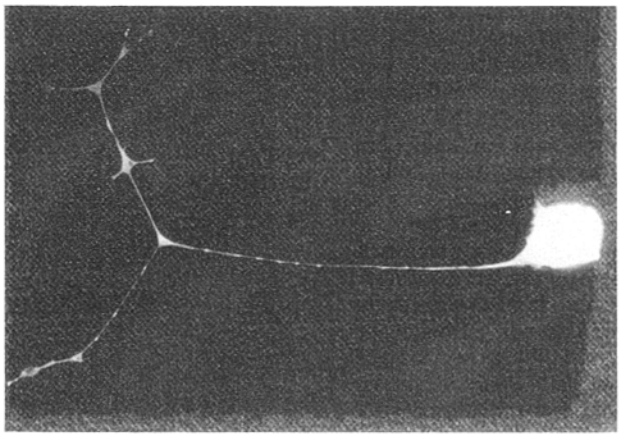

Figure 8. Visualization of the injected antibody HD in the PC12 cell 1 h after injection. The PC12 cell was injected with antibody HD (20 mg/ml), incubated for 1 h, fixed with formaldehyde, and immunostained with the fluorescein-labeled antibody against rabbit IgG. The antibody filled the cytoplasm and neurites of the injected cell. Bar, 30 μm.

developed neurites and therefore the antibody does not change significantly the neurite outgrowth.

To rule out the possibility that the HD antibody does not react with kinesin in PC12 cells, the antibody was tested on immunoblots of differentiated and nondifferentiated PC12 cell extracts. The antibody reacted to a protein with an electrophoretic mobility identical to the kinesin heavy chain from brain (data not shown).

Discussion

The aim of the experiments described in this paper was to

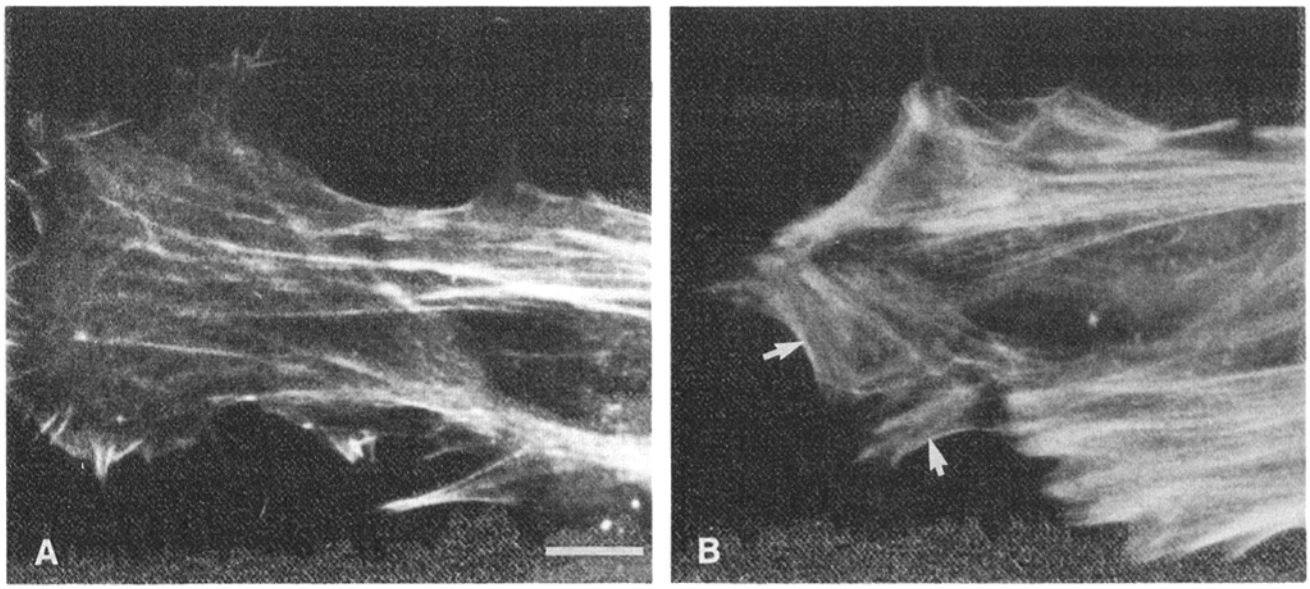

Figure 7. Actin filaments in fibroblasts injected with preimmune IgG or antibody HD. Cells were injected with either preimmune IgG or antibody HD at 20 mg/ml, and 4 h later fixed and stained with rhodamine-phalloidin to reveal actin microfilaments. Arrows point short actin microfilaments bundles at the front edge typical for the HD-injected cells. *A*, preimmune IgG-injected cell; *B*, fibroblast, injected with HD antibody. Bar, 10 μm.

understand the mechanisms of microtubule involvement in control of cell shape and pseudopodial activity in cultured fibroblasts. More specifically, we wanted to know whether any members of the kinesin superfamily play a role in this process. As a tool for inhibiting these proteins, we used injections of the affinity-purified polyclonal antibody against the motor domain of kinesin. From our point of view, there are two advantages to this approach. First, one can use systems where the cell behavior is well known, yet genetic knockout experiments are impossible or difficult. Second, considering the plethora of kinesinlike motors that have recently been discovered (Endow, 1991; Goldstein, 1991), a polyclonal antibody against the motor domain of kinesin, the region that is highly homologous between the different members of the superfamily, is the best starting tool for analyzing the role of motors in the microtubule-dependent control of cell polarity, since it most likely blocks the function of multiple motors. This is particularly important considering the possibility that multiple motors with different primary structure may perform similar functions within the cell so knocking out one of the motors may produce no phenotype (for example see Saunders and Hoyt, 1992).

Human diploid fibroblasts that were used in these experiments are rather extended cells displaying prominent shape alterations in response to microtubule disruption. It was important to confirm that HD injections really interfered with organelle transport and distribution in these particular cells. The remarkable organelles in fibroblasts are mitochondria which can be easily revealed with rhodamine-123 vital staining. It has been shown that mitochondria coaligned with microtubules in fibroblasts and their distribution depended on microtubule network (Ball and Singer, 1982). Moreover, kinesin has been reported to associate with isolated mitochondria and could be revealed in patches on the mitochondrial surface by immunostaining (Leopold et al., 1992). So, we could expect the distribution of mitochondria in 1,036 fibroblasts to be affected by the HD antibody. Indeed, we found that mitochondria collapsed to the cell center after the injection of antibody HD. Thus, positioning of mitochondria in fibroblasts, like positioning of lysosomes in macrophages (Hollenbeck and Swanson, 1990) and melanosomes in melanophores (Rodionov et al., 1991) depends on the functions of kinesin or related proteins recognized by our antibody. An attractive explanation of our results is that antibody injections selectively switched off motor(s) which directed mitochondria toward the cell periphery leaving unaffected a translocator of the opposite polarity that supported the transport of mitochondria toward cell center. In any case, the experiments showed that organelle positioning and transport in 1,036 fibroblasts were selectively suppressed by HD injections.

The most important result of the present work is that HD injection had a prominent effect on the shape of fibroblasts and on the pseudopodial activity at the leading edge of the cells migrating into the wound. These effects seemed to be highly specific as preimmune IgG injections induced only minor effects on both cell shape and pseudopodial activity. The effects of antibody injections were very similar to the effects of nocodazole treatment. Like nocodazole, HD injections significantly decreased cell asymmetry, and both treatments inhibited pseudopodial activity at the leading edge (for nocodazole effects see Bershadsky et al., 1991) and changed the morphology of the microfilament system. However, unlike the microtubule-depolymerizing drugs, HD injections had no effect on microtubule distribution. This result shows an important role of kinesin and/or related translocator proteins in the maintenance of asymmetric cell shape and in polarization of pseudopodial activity, which are closely related to the ability of a cell for a directed migration and suggest that these processes may critically depend on microtubule-mediated organelle transport.

An apparently different result was obtained in the experiments with PC12 cells, where injection of the HD antibody did not have any effect on neurite outgrowth. However, we should stress that it does not necessarily mean that microtubule motors are not involved in neurite elongation in PC12 cells. For example, Ferreira et al. (1992) showed that antisense nucleotides to kinesin heavy chain inhibited neurite elongation in cultures of rat hippocampal neurons. If this is the case for PC12 cells as well, it is possible that some other member of kinesin superfamily rather than kinesin per se is involved in generation of neuronal polarity and this member probably is not inhibited by our antibody.

At present, we do not know the identity of the organelles whose transport might be essential for fibroblast shape and pseudopodial activity. Certain models of cell locomotion involve the addition of new membrane at the leading edge (for review see Singer and Kupfer, 1986); this edge was reported to be the primary site of microtubule-dependent incorporation of membrane marker protein (Bergmann et al., 1983; Rogalski et al., 1984). HD injections could probably stop this membrane addition and thus suppress pseudopodial activity and cell locomotion.

Another possibility is that microtubule-associated organelle transport is needed to maintain the dynamic organization of actin filament system, especially, the extension of leading lamella and pseudopodia. A correlation between positions of microtubular ends and formation of actin-rich ruffles and initial cell–substrate contacts at the leading edge of chick fibroblasts was observed by Rinnerthaler et al. (1988). Motor-based anterograde movement of vesicles may take a part in the transfer of actin and actin-binding proteins to the leading edge region. Rapidly moving actin-containing parcels in growing processes of nerve cells were described by Hollenbeck and Bray (1987). On the other hand, kinesin-mediated transport of mitochondria toward the active edge may provide energy for actin polymerization or other conditions required for pseudopodia extension. In fact, structures made of actin filaments are highly sensitive to ATP deprivation (Svitkina et al., 1986). It is also possible that definite membranous organelles that are to be transported by microtubule motors control the local calcium concentration or other signals regulating pseudopodia extension. At last, we should also take into consideration that HD injections may have indirect effect on cell shape through the collapse of vimentin intermediate filaments (Gyoeva and Gelfand, 1991).

All of these specific possibilities remain to be tested. In any case, our data indicate that microtubule motors are essential for development of maximal pseudopodial activity in one definite direction that may in turn determine the shape and motility of the cell. This conclusion suggests that the role of microtubules in maintaining cell polarity is not just to provide a rigid framework to maintain cell extension, but to organize directed delivery of cell components necessary for the activity of the leading edge. Two important questions clearly

remain to be answered. First, what is the nature of the material/signal required for the activity of the leading edge and second, which particular motors are responsible for its polar delivery?

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